Lasers in Surgery and Medicine 20:56–63 (1997)

Effects of Photostimulation on Wound Healing in Diabetic Mice

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Background and Objective: **Low-level laser irradiation at certain fluences and wavelengths can enhance the release of growth factors from fibroblasts and stimulate cell proliferation in vitro. We evaluated whether low-level laser irradiation can improve wound healing in diabetes mellitus.**

Study Design/Materials and Methods: **Genetically diabetic mice (C57BL/Ksj/db/db) were used as the animal model for this wound healing study. The experimental animals were divided among four groups: negative control, positive control (topical basic fibroblast growth factor [bFGF] on wound), laser therapy group; and a combination group of laser therapy and topical bFGF. An argon dye laser (Lexel Auora Model 600) at a wavelength of 630 nm and an output of 20 mW/cm^2 was used as the light source. The speed of wound closure and histological evaluation were used to analyze the experimental results.**

Results: **Laser irradiation enhanced the percentage of wound closure over time as compared to the negative control group (58.4 ± 2.6 vs. 40.8 ± 3.4 at day 10 and 95.7 ± 2 vs. 82.3 ± 3.6 at day 20, $P < .01$). Histological evaluation showed that laser irradiation improved wound epithelialization, cellular content, granulation tissue formation, and collagen deposition in laser-treated wounds as compared to the negative control group (6.4 ± 0.16 vs. 3.8 ± 0.13 at day 10 and 12 ± 0.21 vs. 8.2 ± 0.31, $P < .01$).**

Conclusion: **This study of laser biostimulation on wound healing in diabetic mice suggests that such therapy may be of great benefit in the treatment of chronic wounds that occur as a complication of diabetes mellitus. Lasers Surg Med 20:56–63, 1997.**

Key words: growth factors; lasers; biostimulation; wounds; diabetes; rodents

INTRODUCTION

Photo-biostimulation has been used for a variety of medical therapies such as wound healing and pain control as well as basic scientific research [1]. Photo-irradiation at low energy levels can generate significant bio-effects which are manifested in biochemical, physiological, and proliferative phenomena in various enzymes, cells, tissues, organs, and organisms. The phenomena of stimulation and inhibition caused by laser light are termed laser biostimulation and bioinhibition, respectively. In vitro data demonstrate that low-level laser irradiation can stimulate cell proliferation [2–7], collagen synthesis [3,4], and the release of growth factors from cells [5,6]. Karu [8] has provided an action spectrum for the biostimulation of the rate of DNA synthesis in HeLa cells, and for the proliferation of bacteria and yeast colonies. These spectra show peaks in the blue (404 and 454 nm), red (620 nm), and near infrared (760 and 830 nm) wavelengths. Hans et al. [7] observed fibroblast proliferation and collagen production using a He-Ne laser (632.8 nm) to irradiate cells in vitro and stated that the power density and exposure time of He-Ne laser irradiation are more important than the total energy dose in photo-bio-

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Accepted for publication 7 December 1995.

modulation. Most studies [9–11] suggest that laser biostimulation occurs at fluences between 0.05 and 10 J/cm^2, whereas fluences above 10 J/cm^2 have bioinhibitory effects. In animal studies, beneficial effects on wound healing have been reported [12,13]. The most commonly stated mechanism for the laser enhancement of wound healing is that laser irradiation stimulates intracellular metabolism and collagen production by fibroblasts [2,8,13]. However, the basic mechanisms of laser biostimulation are poorly understood. The clinical application of laser biostimulation is extremely controversial in the United States. The major reason is that efficacy has not yet been demonstrated unequivocally with properly controlled experiments and clinical trials.

Normal wound healing proceeds in three overlapping phases: inflammation, granulation tissue formation, and matrix formation and remodeling [14]. This process is believed to require the interaction of cells in the dermis and epidermis as well as the activities of chemical mediators released from inflammatory cells, fibroblasts, and keratinocytes. Although the precise interactions of the components constituting normal wound healing are not well understood, a number of conditions such as diabetes mellitus, immunodeficiency, venous stasis, and chemotherapy result in delayed wound healing [15].

Many of the chronic complications of diabetes mellitus involve defects in connective tissue such as poor wound healing, diminished bone formation, and decreased linear growth [16–18]. The wound healing abnormalities of diabetes mellitus result from several causes [19]. When carbohydrates are unavailable to cells for normal aerobic metabolism, oxidation of amino acids for caloric needs results in amino acid and protein depletion. When glycogenolysis and gluconeogenesis fail to provide glucose to meet the energy requirements for fibroblasts and leukocytes, they become dysfunctional, and impaired wound healing results. The poor wound healing of diabetes mellitus has been shown to be associated with decreased amounts of collagen fibrils and collagen production [20,21]. Hyperglycemia interferes with ascorbate transfer into fibroblasts and leukocytes, which also impairs the healing response. Impaired fibroblast and endothelial cell proliferation, epithelialization, decreased collagen deposition, and reduced strength are also all characteristic deficits observed in streptozocin-induced diabetic animals.

Based on our previous in vitro studies, we hypothesized that low-level laser irradiation (LLLI) may have biostimulatory effects on wound healing in vivo. The impaired wound healing of diabetes mellitus may thus provide a sensitive model for studies of the mechanism of low-level laser therapy. In order to limit animal variation, genetically diabetic mice were chosen for this study. This study was undertaken to investigate whether LLLI can enhance wound healing in an animal model as compared to the use of cytokines.

MATERIALS AND METHODS

Animals and Wounding

Forty genetically diabetic mice (C57BL/Ksj/db/db) purchased from Jackson Laboratory (Jackson, ME) were used for this study. All animal care and surgery were carried out in accordance with an approved protocol reviewed by Rochester General Hospital's Institutional Animal Care and Use Committee. The mice were anesthetized with intraperitoneal pentobarbital solution (60 mg/kg), their dorsal hair was clipped, and the skin was disinfected. Two full-thickness circular wounds were created on the dorsum of each mouse using a skin punch biopsy instrument (6 mm diameter). The two wounds were separated by about 1 cm between the edges of the punch biopsies. The wound areas were immediately covered with a semipermeable, transparent dressing (Tegaderm®; 3M Corp., St. Paul, MN). The animals tolerated the wounding procedure without any problems. None of the wounded animals had significant changes in food consumption or weight loss. The transparent dressing remained in place until the wounds healed. The experimental mice were equally divided among four groups: negative control (0.1 ml 5% polyethylene glycol (PEG) in phosphate-buffered saline (PBS) per wound under the dressing); positive control (topical 1 μg basic fibroblast growth factor [bFGF] in 0.1 ml 5% PEG in PBS per wound under the dressing); laser therapy group (laser irradiation at 5 J/cm^2 per wound); and the combination group of laser irradiation and topical bFGF (laser irradiation at 5 J/cm^2 + 1 μg bFGF in 0.1 ml 5% PEG in PBS per wound). The treatments were applied daily for 4 days starting 6 hours after wounding.

The Percentage of Wound Closure

The two perpendicular diameters of each wound were measured using a electronic caliper at 5, 10, and 20 days post-wounding. The percentage of wound closure was calculated for each wound based on the total area of the wound. It

TABLE 1. Criteria for Scoring Histologic Sections*

Score	Parameter	Criteria
1–3	Epithelialization	None to very minimal
	Cellular content	None to very minimal (mainly inflammatory cells)
	Granulation tissue	None to sparse amount at wound edges
	Collagen deposition	None
	Vascularity	None
4–6	Epithelialization	Minimal (less than half of wound diameter) to-moderate (more than half of wound diameter)
	Cellular content	Predominantly inflammatory cells, few fibroblasts
	Granulation tissue	None to thin layer at wound center, thicker at wound edges
	Collagen deposition	Few collagen fibers
	Vascularity	Few capillaries
7–9	Epithelialization	Completely epithelialized; thin layer
	Cellular content	More fibroblasts, still with inflammatory cells
	Granulation tissue	7, sparse at wound center, mainly adipose tissue underneath epithelium; 8, thin layer at wound center, few collagen fibers; 9, thicker layer, more collagen
	Vascularity	Moderate neovascularization
10–12	Epithelialization	Thicker epithelial layer
	Cellular content	Predominantly fibroblasts
	Granulation tissue	Uniformly thick
	Collagen deposition	Moderate-to-extensive collagen deposited, but less mature as compared to collagen of unwounded skin margin
	Vascularity	Extensive neovascularization
13–15	Epithelialization	Thick epithelium
	Cellular content	Fewer number of fibroblasts in dermis
	Granulation tissue	Uniformly thick
	Collagen deposition	Dense, organized, oriented collagen fibers
	Vascularity	Well-defined capillary systems

*The scoring will be based on the degree of re-epithelialization, cellular invasion, granulation tissue formation, collagen deposition, and vascularity utilized by previous investigators [53–55].

was expressed by the following formula: % closure = [(area on day 0 – open area on final day)/area on day 0] × 100. Five mice from each group were sacrificed on day 10 and day 20. The wound tissues were harvested for histologic study.

Histologic Evaluation

Histologic evaluation was performed by two independent observers. Each slide was given a histologic score ranging from 1 to 15. The scoring was based on the degree of re-epithelialization, cellular invasion, granulation tissue formation, collagen deposition, and vascularity according to the criteria stipulated in Table 1.

Laser Irradiation

A Lexel Aurora Argon Dye laser (Model, 600 Dye laser, Fremont, CA) was used at a wavelength of 630 nm for irradiation. The precise measurement of the laser wavelength was determined via spectrometer (RS Instruments, San Jose, CA). The power output was measured using a laser power meter (Molectron Detector Inc. Portland, OR). The power density of laser irradiation was maintained at 20 ± 0.8 mW/cm^2 within a 2-cm-diameter spot. The wounds were irradiated for periods of 250 sec at each treatment session and received a fluence of 5 J/cm^2.

RESULTS

The percentage of wound closure and the histologic scoring data are shown in Table 2. The histology shows that most wounds treated with laser irradiation appeared to have a moderate degree cell proliferation, a predominance of inflammatory cells, and few fibroblasts at day 10. A thin layer of granulation tissue existed at the wound center, and this generally became thicker at the wound edges by day 10 (Fig. 1). A uniformly thick epithelial layer with many fibroblasts, extensive neovascularization, and moderate-to-extensive collagen deposition were observed at day 20 (Fig. 2). In contrast, the negative control wound appeared to have minimal cellular infiltrates and less granulation tissue in the wounds at day 10, and a thin layer of epithelial and granulation tissue with few collagen fibers at day 20. The histo-

TABLE 2. Wound Closure and Histologic Scoring†

Groups	% wound closure		Histologic score	
	Day 10	Day 20	Day 10	Day 20
Negative control	40.8 ± 3.4	82.3 ± 3.6	3.8 ± 0.13	8.2 ± 0.31
Positive control	56.3 ± 2.9*	89.0 ± 3.6*	6.0 ± 0.21*	11.2 ± 0.3*
Light + bFGF	57.5 ± 2.9*	92.3 ± 3*	5.4 ± 0.16*	10.7 ± 0.41*
Light	58.4 ± 2.6*	95.7 ± 2*	6.4 ± 0.16*	12.0 ± 0.21*

†Negative control: 0.1 ml 5% polyethylene glycol (PEG) in PBS per wound; positive control: 1 μg bFGF in 0.1 ml 5% PEG in PBS per wound; light + bFGF: laser irradiation at 5 J/cm^2 + 1 μg bFGF in 0.1 ml 5% PEG in PBS per wound; light: laser irradiation at 5 J/cm^2 per wound + 0.1 ml 5% PEG in PBS per wound. The results of % wound closure and histologic score were represented as mean ± SEM in each group. Evaluation of histological score was based on Table 1.

*$P < .05$ as compared to negative control.

logic results for the positive control and combination treatment of laser irradiation and bFGF appeared to be similar to the wounds that received laser treatment alone.

The percentage of wound closure after laser irradiation at a fluence of 5 J/cm^2 was significantly enhanced ($P < .01$) as compared to the negative control group both at day 10 and day 20. Histologic evaluation of the laser treatment, positive control (bFGF), and the combination group wounds all showed a significant improvement ($P < .01$) in wound healing as compared to the negative control group at day 10 and day 20 (Table 2; Fig. 1). There was no difference in the degree of wound closure between the laser group and the positive control group. Treatment with the combination of bFGF and laser irradiation also improved wound healing as compared to the negative controls but did not show any apparent enhancement in healing when compared to laser treatment alone or the positive control group.

DISCUSSION

Since Mester et al. [22] reported that low-energy laser treatment had a stimulatory effect on wound healing, low-level laser therapy has been used successfully to treat trophic ulcers and indolent wounds of diverse etiologies when traditional treatments had little effect [23,24]. In addition, LLLI has been reported to accelerate bone repair following fracture or radiation-induced osteonecrosis [25–27]. However, conflicting results have been reported as to the effect of low-level laser therapy on wound healing [28–31]. The reason for these discrepancies may be due to widespread variability in the choice of experimental animal utilized, the type and size of wound, the method of assessment of results, differences in laser wavelengths and fluences, and the conditions of treatment.

The db/db mouse model provides an excellent analogy to human diabetes. These mice are obese and develop hyperglycemia that is resistant to insulin. Complications seen in human diabetes such as peripheral neuropathy, microvascular lesions, basement membrane thickening, glomerular filtration abnormalities, immune complex deposition, and immunodeficiency have all been observed in the db/db mouse [32]. Only the homozygous (db+/db+) animals develop diabetes, whereas the heterozygous (db+/+m) littermates show no signs of diabetes or obesity. Previously published work on the impairment of wound healing in diabetic animals was performed using the cytotoxic drugs alloxan or streptozocin to induce the diabetic state in genetically intact animals. These treatments result in decreases in collagen synthesis in subcutaneous chambers and decreases in the tensile strength of incisions [33,34]. A confounding factor in streptozocin-induced diabetes is that the drug is toxic not only to pancreatic beta cells but also to the liver, renal tubules, and the exocrine pancreas [35]. In addition, alloxan and streptozocin alter macrophage phagocytosis and depress T-cell function [36]. Since C57BL/KsJ/db/db mice have a clinically relevant and reproducible model of impaired wound healing, we chose this genetically diabetic animal model in an effort to provide more realistic and reliable results of laser therapy on wound healing which would simulate the clinical state of maturity-onset diabetes.

This study demonstrates that LLLI enhances wound healing in diabetic mice as evidenced by the percentage of wound closure and the histological evaluation of wounds at day 10 and day 20 in the laser-treated animals as compared to the control

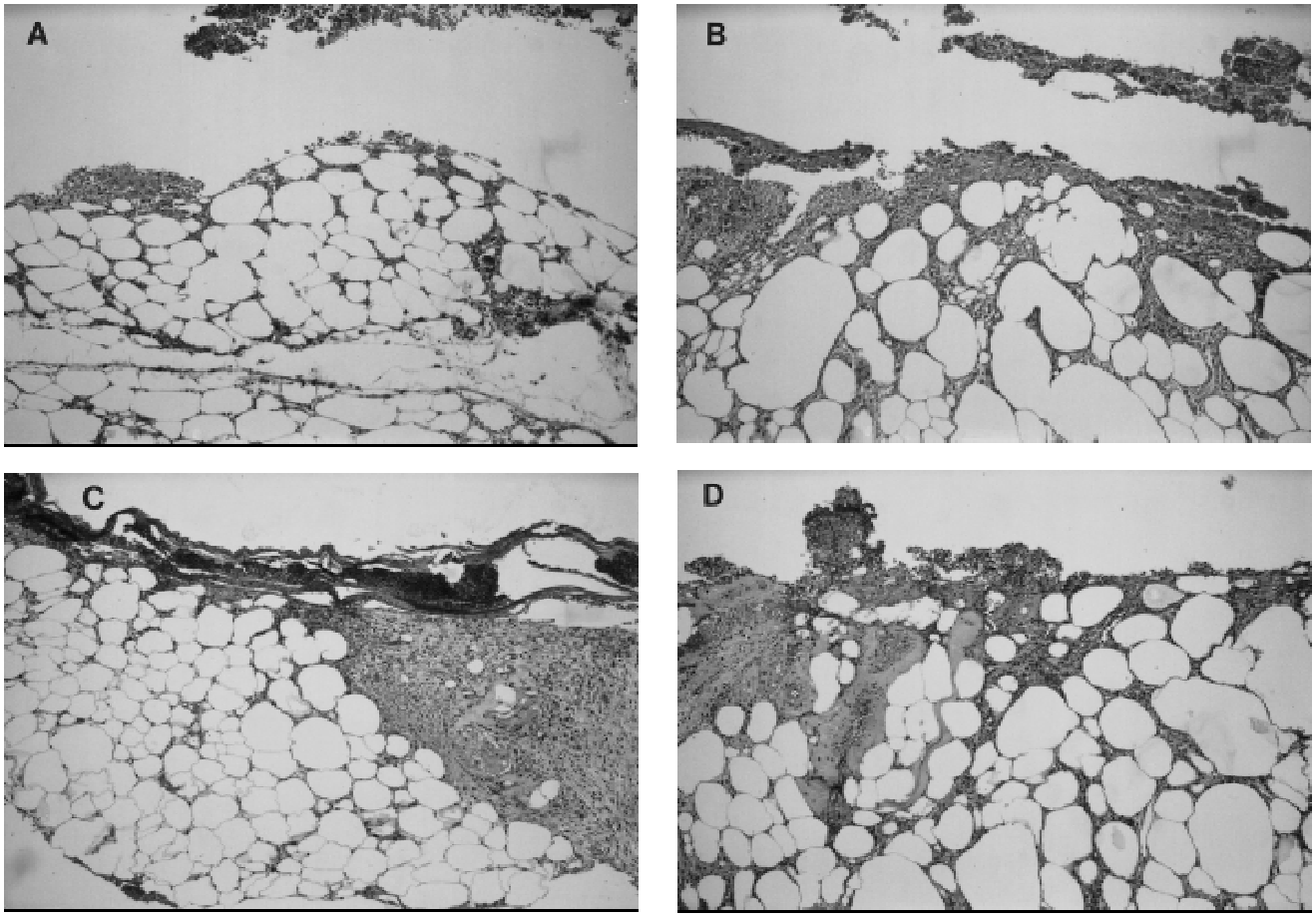

Fig. 1. Hematoxylin and eosin-stained sections of skin wounds on genetically diabetic mice at day 10. **A:** Negative control. **B:** Positive control (1 μg bFGF). **C:** Laser treatment. **D:** Laser irradiation plus bFGF treatment. The wounds treated with laser irradiation appeared to have a moderate degree cell proliferation, a predominance of inflammatory cells, and few fibroblasts. A thin layer of granulation tissue exists at the wound center, and this generally became thicker at the wound edges by day 10.

group. This effect may involve a variety of photobiostimulating mechanisms. One current theory of the mechanism of photostimulation is that the mitochondria are the photoacceptors for visible light energy. The absorption of light by the respiratory chain components may cause a short-term activation of the respiratory chain and oxidation of the NADH pool. This leads to changes in the redox status of both the mitochondria and the cytoplasm. Activation of the electron-transport chain results in an increase of promotive force, an increase in the electrical potential of the mitochondria membrane and the ATP pool, the alkalization of cytoplasm, and finally, the activation of nucleic acid synthesis. In our previous in vitro study [5,6], we found that low-level laser energy at certain fluences can modulate cell proliferation and that this result is correlated with the release of growth factors from fibroblasts. Therefore, the photo-biostimulating effect on wound healing may involve the enhancement of growth factors release from irradiated cells, and fibroblast growth factors in particular.

Growth factors have been shown to stimulate angiogenesis [37,38], extracellular matrix production and degradation [39–45], and cytokine release [46,47]. Attempts to improve the healing of full-thickness wounds in animals with growth factors have resulted in observations which vary from slight improvement [48–50] to no effect at all [51,52]. Animals with impaired healing induced by drugs may be more prone to variations in the degree of impairment between individuals than animals in which the impairment has a genetic basis. Several investigators have improved isolated aspects of the normal

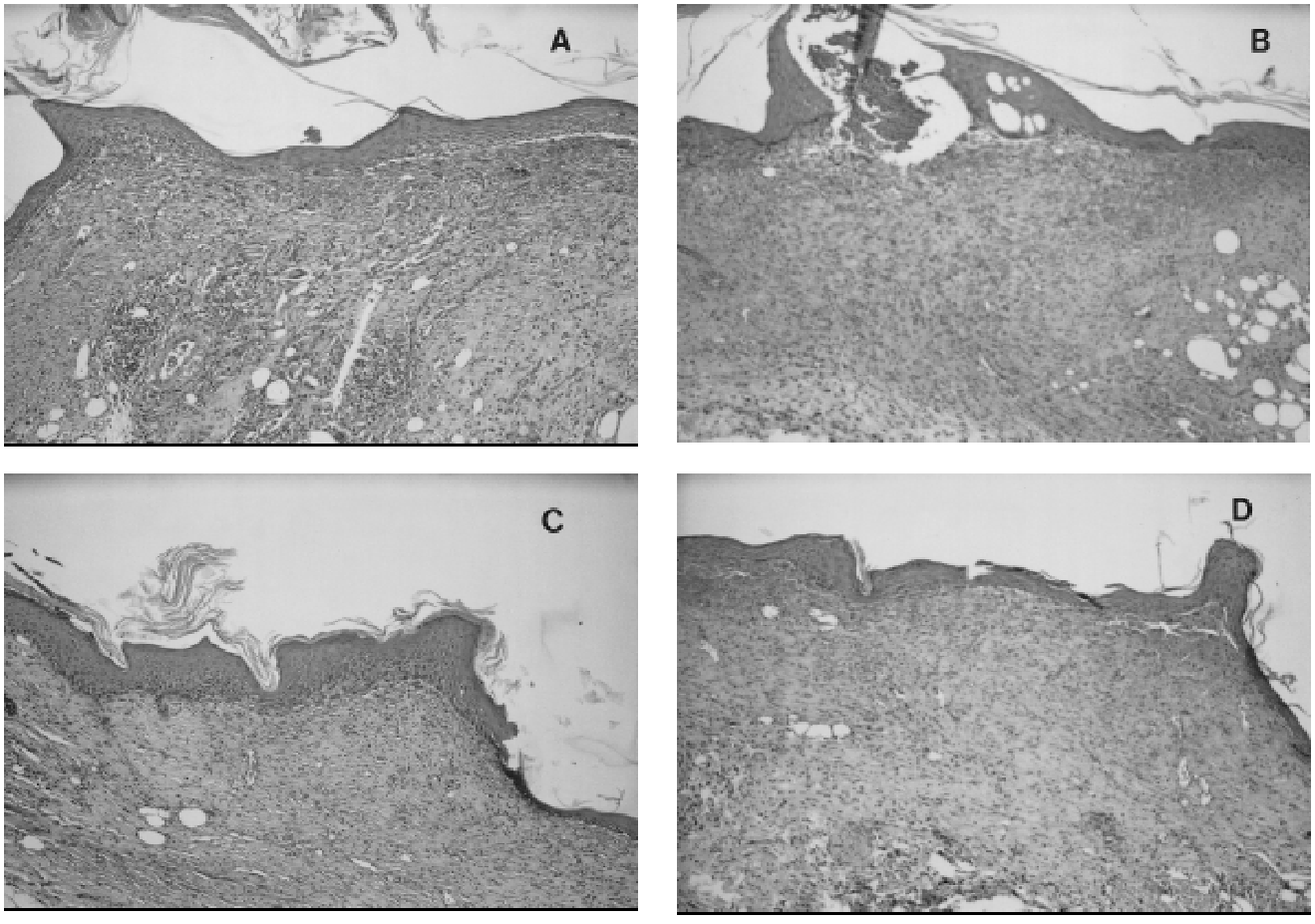

Fig. 2. Hematoxylin and eosin-stained sections of skin wounds on genetically diabetic mice at day 20. **A:** Negative control. **B:** Positive control (1 μg bFGF). **C:** Laser treatment. **D:** Laser irradiation plus bFGF treatment. A uniformly thick epithelial layer with many fibroblasts, extensive neovascularization and moderate to extensive collagen deposition are all observed in the wound treated with laser at day 20.

healing process, but the beneficial effects do not appear to change the overall time required for complete wound repair. One explanation for the modest effects seen after the application of growth factors to wounds in normal animals may be that the healing process already proceeds at a near-optimal rate. In this study, both the positive control group, which was treated with 1 μg bFGF per wound, and the laser treatment group demonstrated enhanced wound healing when compared to the negative control. However, laser irradiation appeared to improve wound healing to a greater extent than bFGF treatment alone. Combined treatment with bFGF and laser irradiation also improved wound healing as compared to the negative control. However, there was no apparent increase in this effect when this was compared to the laser treatment alone or the positive control group (i.e., bFGF treatment alone). One possible explanation for this result is that the application of either laser irradiation or growth factor may already have caused wound healing to proceed at a near-optimal rate, and that combining agents fails to increase the rate induced by either agent alone beyond this rate.

CONCLUSIONS

Our studies have shown that low-level laser irradiation not only stimulates cell proliferation and the release of growth factors in vitro but also enhances wound healing in diabetic mice. This study of laser biostimulation on wound healing in diabetic mice suggests that such therapy may be of benefit in the treatment of chronic wounds such as those which occur as a complication of diabetes mellitus. Further studies of the dose dependence

of laser energy on wound healing and the mechanism of photobiostimulation are warranted.

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